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Highlights

FRONTLINE:

Broad T cell immunity to the LcrV virulence protein is induced by targeted delivery to DEC-205/CD205-positive mouse dendritic cells

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There is a need for a more efficient vaccine against the bacterium *Yersinia pestis*, the agent of pneumonic plague. The F1-LcrV (F1-V) subunit vaccine in alhydrogel is known to induce humoral immunity. In this study, we utilized DC to investigate cellular immunity. We genetically engineered the LcrV virulence protein into the anti-DEC-205/CD205 mAb and thereby targeted the conjugated protein directly to mouse DEC-205$^+$ DC *in situ*. We observed antigen-specific CD4$^+$ T cell immunity measured by intracellular staining for IFN-γ in three different mouse strains (C57BL/6, BALB/c, and C3H/HeJ), while we could not observe such T cell responses with F1-V vaccine in alhydrogel. Using a peptide library for LcrV protein, we identified two or more distinct CD4$^+$ T cell mimetopes in each MHC haplotype, consistent with the induction of broad immunity. When compared to nontargeted standard protein vaccine, DC targeting greatly increased the efficiency for inducing IFN-γ-producing T cells. The targeted LcrV protein induced antibody responses to a similar extent as the F1-V subunit vaccine, but Th1-dependent IgG2a and IgG2c isotypes were observed only after anti-DEC-205:LcrV mAb immunization. This study sets the stage for the analysis of functional roles of IFN-γ-producing T cells in *Y. pestis* infection.

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Introduction

Yersinia pestis, the causative agent of plague or Black Death, is again drawing attention due to its potential use as a biological weapon [1, 2]. Bubonic plague is the most common clinical form of disease and is characterized by swollen lymph nodes (buboes) following bites by infected fleas. If untreated, it can progress to the life-

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14. ABSTRACT

There is a need for a more erfficient vaccine against the bacterium Yersinia pestis, the agent of pneumonic plague. The F1-LcrV (F1-V) subunit vaccine in alhydrogel is known to induce humoral immunity. In this study, we utilized DC to investigate cellular immunity. We genetically engineered the LcrV virulence protein into the anti-DEC-205 CD205 mAb and thereby targeted the conjugated protein directly to mouse DEC205+ DC in situ. We observed antigen-specific CD4+ T cell immunity measured by intracellular staining for IFN-gamma in three different mouse strains (C57BL/6, BALB/c, and C3F/HeJ), while we could not observe such T cell respinses with F1-V vaccine in alhydrogel. Using a peptide library for LcrV protein, we identified two or more distinct CD4+ T cell mimetopes in each MHC haplotyupe, consistent with the induction of broad immunity. When compared to nontargeted standard protein vaccine, DC targeting greatly the efficiency for inducingIFN-gamma-producing Tcells. The targeted LcrV protein induced antibody responses to a similar extent as the F1-V subunit vaccine, but Th1-dependent IgG2a and IgG2c isotypes were observed only after anti-DEC-205:LcrV mAb immunization. This study sets the stage for the analysis of functional roles of IFN-gamma-producing T cells in Y. pestis infection.

15. SUBJECT TERMS

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threatening septicemic and pneumonic forms of plague. The latter form also occurs *via* contaminated respiratory droplets, or even by artificially generated aerosols containing *Y. pestis*, and is particularly dangerous because of its high contagiousness and mortality [1]. There is a need to develop more effective vaccines for pneumonic plague.

Prior vaccines against *Y. pestis* that have been used in man are either attenuated or killed organisms, and are insufficiently effective against pneumonic diseases [3]. In a murine model, a subunit vaccine composed of recombinant F1 and LcrV protein (F1-V) has been demonstrated to induce protective responses [4]. F1 antigen is an extracellular capsular antigen, encoded on a 110-kb plasmid (pMT-1) and exerting anti-phagocytic activity [5]. Protective immunity *via* F1 has been shown in a murine model, and in humans, F1 elicits strong antibody responses following immunization with purified native antigen [6, 7]. The other component of the current subunit vaccine, LcrV, is a secreted multifunctional protein encoded on a 70-kb plasmid (pCD1). LcrV regulates the delivery of other effector molecules of *Y. pestis* or Yops into host cells upon contact *via* a type III secretion system [8, 9]. LcrV is also reported to have immunosuppressive potential by stimulating IL-10 secretion [10]. Although LcrV alone has been shown to protect mice against infection with either $F1^+$ or $F1^-$ strains, the F1-V combination provides better protection than either subunit vaccine alone, and moreover, it protects mice against pneumonic plague [11–13].

Recent studies have indicated that besides humoral immunity, the induction of cellular immunity might be an important goal for a plague vaccine. $CD4^+$ T cells are able to produce high levels of Th1 cytokines such as IFN-γ, which can activate macrophages to kill intracellular pathogens, and Th cells also contribute to antibody-based immunity. In addition, $CD4^+$ T cells can exert cytolytic activity on MHC class II-bearing targets [14]. It was first noted that treatment of mice with exogenous IFN-γ plus TNF-α inhibited the multiplication of *Y. pestis in vivo*, thereby providing protection against intravenous challenge against 10 minimum lethal doses of $LcrV^+$ *Y. pestis* KIM [15]. Likewise, LcrV antigen co-encapsulated with IFN-γ induced higher antigen-specific systemic immune responses [16]. Moreover, STAT4-deficient mice, which have low levels of IFN-γ production, were poorly protected from *Y. pestis* GB by the s.c. route, despite producing as high levels of serum antibody as wild-type controls [17].

A protective role of $CD4^+$ T cells in *Y. enterocolitica* infection was recently demonstrated by Smiley and colleagues [18], where three LcrV-specific CD4 epitopes were identified that are presented in the context of the murine $I\text{-}A^b$ MHC class II molecule. This group further showed that the transfer of *Y. pestis*-primed T cells to naive B cell-deficient mice (μMT) protected against attenuated *Y. pestis* (KIM D27) intranasal challenge [19]. In another study, Parent *et al.* [20] concluded that IFN-γ, TNF-α and nitric oxide synthase 2 are key elements of cellular immunity during pulmonary *Y. pestis* (KIM D27) infection. Therefore, an effective plague vaccine may need to prime not only humoral immunity but also strong Th1-type cellular immunity.

In this study, we targeted the LcrV virulence protein to dendritic cells (DC), which are potent and specialized antigen-presenting cells, with the aim of generating more effective Th cells. DC are known as "nature's adjuvants", and various potential strategies to exploit DC in vaccine design have been suggested [21]. Recent studies provide a new avenue to DC-based vaccines by using an anti-mouse DC monoclonal antibody (mAb), specifically anti-DEC-205/CD205 mAb, to target vaccine antigens directly to DC *in situ* [22–24]. Antigens incorporated within anti-DEC-205 mAb are efficiently and selectively targeted to DC, leading to greatly enhanced presentation to T cells when compared to nontargeted antigen. This targeting strategy improves T cell vaccination, *e.g.* intensified and protective CD4 T cell immunity is induced to HIV gag p24 and p41 proteins by DEC-205 targeting and this provided protection against an airway challenge with recombinant vaccinia-gag virus [23].

Using anti-DEC/LcrV fusion mAb together with DC maturation stimuli, we observed strong and broad antigen-specific Th1-type $CD4^+$ T cell immunity as well as humoral immunity including high titers of Th1-type antibodies, which was not observed with the recombinant subunit F1-V vaccine. This study provides a new way to study the functional roles of Th1-type T cells in plague and suggests a way to the development of vaccines that include strong cell-mediated as well as humoral immunity.

Results

Generation of fusion mAb of LcrV protein engineered into anti-DEC-205

To target LcrV protein to DC directly *in vivo*, the full-length LcrV sequence was first codon-optimized to improve expression and cloned in frame into the heavy chain of anti-mouse DEC-205 mAb as described [25]. Due to the insertion of LcrV, which has a mass of 37 kDa, the heavy chain of the chimeric mAb was detected at ~97 kDa, following SDS-PAGE and either Coomassie staining or Western blotting (Fig. 1A, C).

To verify that the chimeric mAb bound properly to mouse DEC-205 receptor, a stable Chinese hamster ovary (CHO) cell transfectant, expressing mouse

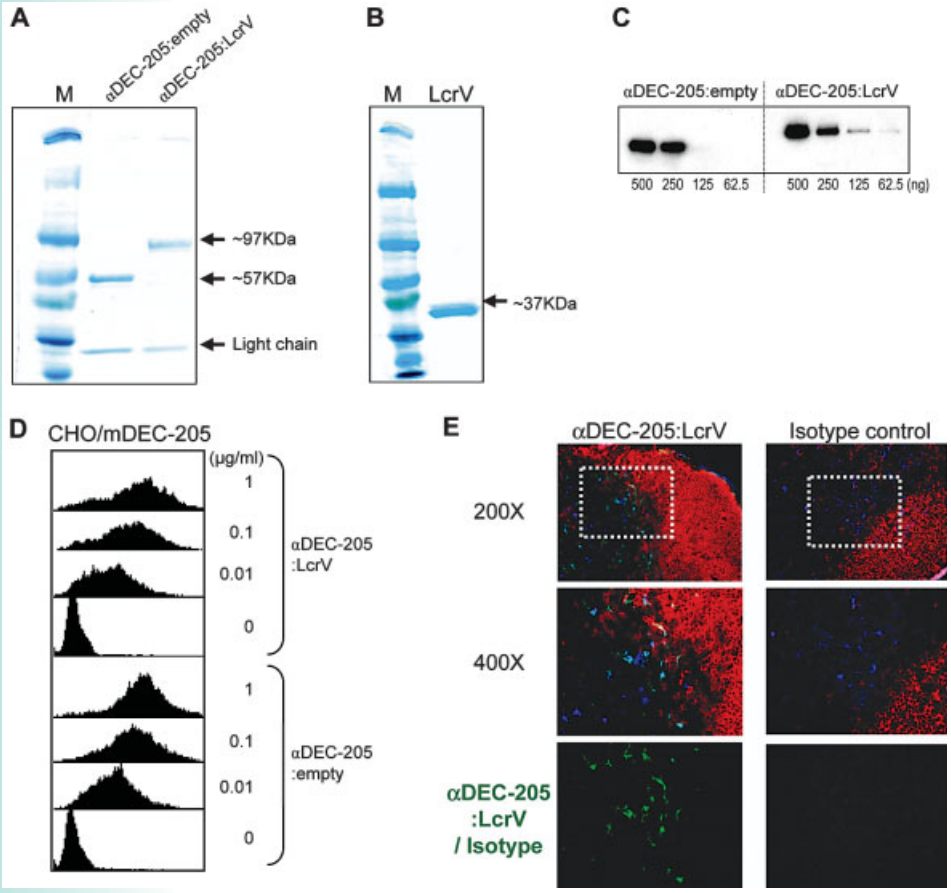

Figure 1. Characterization of a fusion anti-DEC-205 (αDEC-205) mAb and LcrV protein and its *in situ* targeting to DEC-205$^+$ cells in the T cell areas of lymph node; M, molecular weight standards (kDa). (A, B) Coomassie Blue staining of fusion mAb (A) and LcrV protein (B). (C) Western blotting of unconjugated mouse IgG1 (αDEC-205:empty) and αDEC-205:LcrV mAb. (D) FACS staining data showing binding capacity of unconjugated αDEC-205 (lower) and αDEC-205:LcrV mAb (upper) to DEC-205 receptor using stable transfectant CHO cells (CHO/mDEC-205). (E) Microscopy to show *in situ* targeting of αDEC-205:LcrV mAb to the inguinal lymph nodes, using Alexa488-conjugated αDEC-205:LcrV mAb (green, left) or isotype control Ab (right) injected into the hind footpads (40 μg/footpad) 6 h earlier. Sections were also stained with PE-conjugated B220 to mark the B cell areas (red) and OLLAS epitope-tagged αDEC-205 antibody (manuscript submitted) to localize endogenous DEC-205$^+$ cells (blue).

DEC-205 receptor on the surface, was stained with various concentrations of the conjugated or non-conjugated mAb. As assessed by FACS, the anti-DEC-205:LcrV mAb bound to DEC-205 receptor as well as the non-conjugated or "empty DEC" mAb (Fig. 1D). In addition, soluble LcrV protein was generated from the stable CHO cell transfectant and purified using an anti-FLAG affinity column (Fig. 1B). To visualize the specificity of targeting *in situ*, lymph node sections were prepared after administration of Alexa488-conjugated anti-DEC-205:LcrV, or isotype control (mouse IgG1) mAb. Staining for the injected mAb was confined to dendritic profiles in T cell area (Fig. 1E, green signal). These profiles were double-labeled for anti-DEC-205 applied to the section (Fig. 1E, blue signal). Taken together, these data indicate the successful generation of the chimeric mAb and specific *in vivo* targeting to DEC-205$^+$ DC.

DEC-205 targeting of LcrV enhances antigen presentation to T cells and broad CD4 T cell immunity *in vivo*

To determine whether the chimeric mAb could induce T cell immune responses, groups of C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice were injected with a single dose of anti-DEC-205:LcrV mAb s.c. in the presence of DC maturation stimulus, which was the combination of 50 μg of poly(IC) and 25 μg of agonistic anti-CD40 mAb. At 14–21 days later, splenocytes were harvested and restimulated with LcrV peptide pools, and the frequency of IFN-γ-secreting antigen-specific CD4$^+$/CD8$^+$ T cells was examined by ELISPOT (data not shown) and intracellular cytokine staining (Fig. 2).

The peptide library that we used was divided into eight pools of 11 overlapping 15-mer peptides staggered every four amino acids (aa) along the entire LcrV sequence. For C57BL/6 mice, CD4$^+$ T cell responses

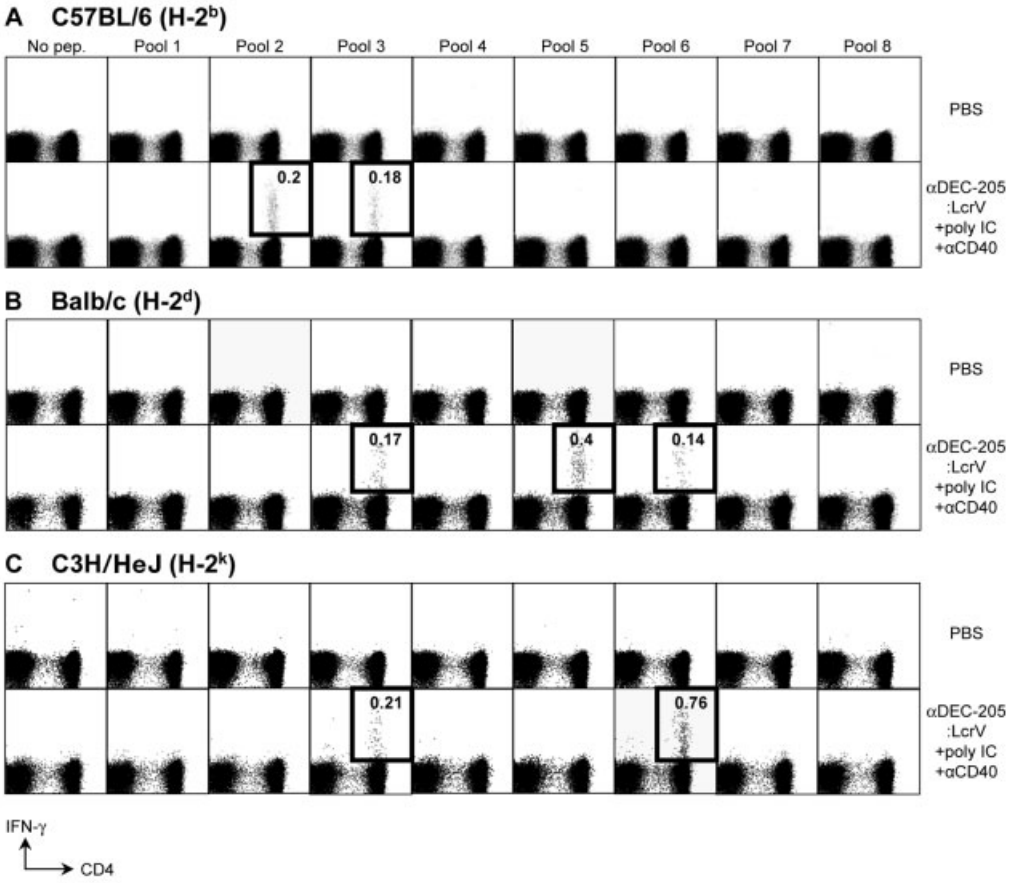

Figure 2. CD4+ T cell responses to a single dose of anti-DEC-205:LcrV (αDEC-205:LcrV) mAb. (A–C) C57BL/6 (A), BALB/c (B), and C3H/HeJ (C) mice were immunized s.c. with αDEC-205:LcrV mAb (10 μg) in the presence of poly(IC) (50 μg) and agonistic anti-CD40 (αCD40) mAb (25 μg) and 2–3 wk later, splenic cells were restimulated with peptide pools 1–8. Percentages of IFN-γ-secreting CD3+/CD4+ T cells were assessed by intracellular cytokine staining (thick-lined boxes). These experiments were repeated three times in each strain with similar results (see Table 2).

were noted to peptides in pool 2 and 3. For BALB/c, peptide pools 3, 5, and 6 were recognized by CD4+ T cells, and for C3H, peptide pools 3 and 6 contained the most reactive epitopes. CD8+ T cell responses were not observed. The reactive peptide pools were then broken down into individual peptides. The reactive mimetope peptides are summarized in Table 1. At least two (C57BL/6 and C3H) and three different mimetopes (BALB/c) were identified. We repeated this experiment twice and summarized the frequency of IFN-γ-secreting

Table 1. LcrV mimetope peptides recognized by mice immunized with anti-DEC-205:LcrV mAb[a)]

Strain and MHC haplotype	LcrV peptide pool	Reactive peptide
C57BL/6 H-2b	2	KILAYFLPEDA (aa 73–83)
	3	EFLEFLESSPNTQW (aa 103–113)
BALB/c H-2d	3	TQWELRAFMAV (aa 111–121)
	5	LKIYSVIQAEINKHL (aa 164–178)
	6	KILEKMPQTTIQV (aa 214–226)
C3H H-2k	3	SSPNTQWELRA (aa 107–117)
	6	EEIFKASAEYK (aa 204–214)

a) Different strains of mice were immunized s.c. with anti-DEC-205:LcrV mAb (10 μg) in the presence of poly(IC) (50 μg) and agonistic anti-CD40 mAb (25 μg), and 2–3 wk later, splenic cells were restimulated with peptide pools 1–8. Then the pools were broken down into individual peptides to identify peptide mimetopes recognized by the immune CD4+ T cells.

Table 2. $CD4^+$ T cell responses to LcrV in three different mice strains after a single immunization with anti-DEC-205:LcrV mAb[a)]

Strain and MHC haplotype	% IFN-γ^+ cells in $CD4^+$ T cells	
	+ peptide	– peptide
C57BL/6 H-2^b	0.19 ± 0.035	0.014 ± 0.005
BALB/c H-2^d	0.36 ± 0.045	0.023 ± 0.006
C3H H-2^k	1.29 ± 0.34	0.03 ± 0.01

[a)] Different strains of mice were immunized s.c. with anti-DEC-205:LcrV mAb (10 μg) in the presence of poly(IC) (50 μg) and agonistic anti-CD40 mAb (25 μg), and 2–3 wk later, splenic cells were restimulated with peptide pools 1–8 (reactive peptides: C57BL/6, KILAYFLPEDA; BALB/c, LKIYSVIQAEINKHL; C3H/HeJ, EEIFKASAEYK). The data shown are mean values for IFN-γ-secreting $CD4^+$ T cells in three experiments without or with restimulation with the dominant recognized peptide.

$CD4^+$ T cells in Table 2. These data indicated that the targeting of LcrV protein to DC induced a broad immune response by $CD4^+$ T cells in several MHC haplotypes.

DEC-205 targeting increases the efficiency of inducing LcrV-specific CD4 T cell responses

To determine the efficiency of targeting strategy, increasing doses of anti-DEC-205:LcrV mAb in the presence of poly(IC) and anti-CD40 were administered into C57BL/6 (data not shown) or BALB/c mice and 14 days later, the frequency of IFN-γ- and/or IL-2-secreting LcrV-specific $CD4^+$ T cells was measured. In comparison, soluble nontargeted LcrV protein with poly(IC) and anti-CD40 as well as F1-V recombinant protein with alhydrogel as an adjuvant were also included (Fig. 3A). The data showed an increased frequency of IFN-γ- and/or IL-2-secreting $CD4^+$ T cells with anti-DEC-205:LcrV mAb immunization in mice, while nontargeted soluble LcrV protein required at least 100 μg to induce antigen-specific T cell responses. Moreover, similar T cell responses were not observed in mice immunized with F1-V adsorbed in alum adjuvant. These data demonstrated that the targeting strategy increases the efficiency of eliciting IFN-γ-producing T cell responses. We also tested different peptide doses during the immune assay and determined that most T cells responded to antigen at 0.2 μM peptide or higher (Fig. 3B). Based on the data, we decided to use 10 μg of anti-DEC-205:LcrV mAb, 100 μg of soluble protein, or 10 μg of F1-V recombinant protein for immunization, and 2 μM of reactive peptide for restimulation in this study unless otherwise indicated.

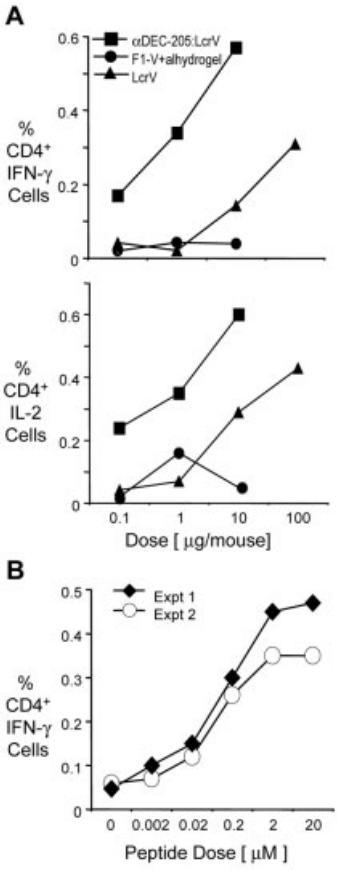

Figure 3. Strong $CD4^+$ T cell responses to a single dose of anti-DEC-205:LcrV (αDEC-205:LcrV) mAb. (A) BALB/c mice were injected s.c. with graded doses of αDEC-205:LcrV mAb, F1-V, or LcrV protein in the presence of adjuvants [poly(IC) and anti-CD40 for αDEC-205:LcrV mAb and LcrV protein, and alhydrogel for F1-V protein]. Two weeks later, splenic T cells were restimulated with LcrV-reactive peptides from pool 5 (BALB/c, LKIYSVIQAEINKHL, aa 164–178) and IFN-γ- or IL-2-secreting $CD4^+$ T cells were assessed by intracellular cytokine staining. Background activity (<0.01%) of PBS- or maturation stimulus alone [25 μg anti-CD40 mAb and 50 μg of poly(IC)]-injected mice was subtracted from the percentage of IFN-γ^+ or IL-2^+ $CD4^+$ cells. Shown is one of two similar experiments with two mice pooled in each experiment. (B) BALB/c mice were immunized with 10 μg of αDEC-205:LcrV mAb with maturation stimuli, and 2 wk later, splenocytes were restimulated with graded doses of LcrV-reactive peptides described in (A). The percentage of IFN-γ^+ $CD4^+$ cells was assessed by intracellular cytokine staining. Shown is one representative experiment of two.

Anti-DEC-205:LcrV immunization enhances $CD4^+$ T cell immunity relative to other immunization approaches

We compared the response to DC-targeted LcrV with other methods of immunization. When we injected soluble LcrV protein, either 10 μg or 100 μg per mouse, with standard adjuvant, complete Freund's adjuvant (CFA), the immune responses were weak compared with

10 μg of DC-targeted anti-DEC-205:LcrV mAb in the presence of poly(IC) and anti-CD40 as adjuvants (Fig. 4A). Again, F1-V recombinant protein adsorbed in alum adjuvant did not induce IFN-γ-secreting $CD4^+$ T cells. In addition, DEC-205 was essential for the enhanced CD4 T cell immunity induced by DC-targeted LcrV protein, because such responses were not observed in DEC-$205^{-/-}$ mice (Fig. 4B). Thus, the data suggested that the targeting of a vaccine protein to DC improves $CD4^+$ T cell immunization relative to other approaches including standard soluble protein immunization as well as F1-V subunit vaccine.

Strong antibody responses to anti–DEC-205:LcrV mAb administration

To study humoral immunity induced by DC-targeted LcrV protein, we tested antibody responses with graded doses of anti-DEC-205:LcrV mAb, soluble LcrV protein, or F1-V. Antibody titers increased with increasing doses of protein (Fig. 5A). Also the titers induced by 10 μg of F1-V with alhydrogel were comparable to those induced by either 10 μg of anti-DEC-205:LcrV or 100 μg of soluble LcrV protein in the presence of poly(IC) and anti-CD40 as adjuvants (Fig. 5A).

Next, anti-LcrV antibody titers for individual IgG isotypes were examined (Fig. 5B). Again, a single immunization of anti-DEC-205:LcrV mAb with adjuvants induced serum levels of each isotype that were comparable to F1-V immunization. The IgG1 and IgG2b isotypes were predominant as described in previous studies [4]. However, the IgG2a (BALB/c) or IgG2c (C57BL/6, data not shown) isotype was observed primarily in mice immunized with anti-DEC-205:LcrV mAb, with little or no IgG2c/IgG2a in the F1-V-immunized group (Fig. 5B). The IgG2c/IgG2a isotypes reflect the influence of Th1-type cells and are consistent with the observation that anti-DEC-205-targeted protein vaccine is a superior way to induce this type of cell-mediated immunity.

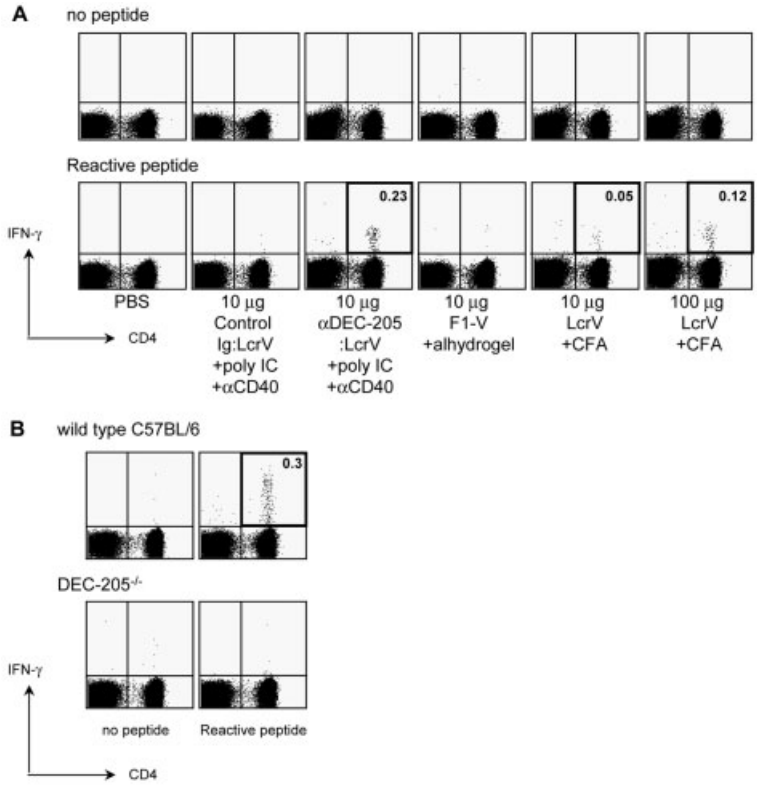

Figure 4. Comparison of $CD4^+$ T cell responses between nontargeted protein immunization and DC-targeted protein. (A) BALB/c mice were injected s.c. with anti-DEC-205:LcrV (αDEC-205:LcrV) mAb (10 μg), F1-V (10 μg), control Ig (10 μg), or LcrV protein (10 or 100 μg) in the presence of adjuvants [poly(IC) and anti-CD40 for αDEC-205:LcrV mAb and control Ig, alhydrogel for F1-V protein, and CFA for LcrV protein]. Two weeks later, splenic T cells were restimulated with LcrV-reactive peptides from pool 5 (BALB/c, LKIYSVIQAEINKHL, aa 164–178) and IFN-γ-secreting $CD4^+$ T cells were assessed by intracellular cytokine staining. Shown is one of two similar experiments with two mice pooled in each experiment. (B) C57BL/6 or DEC-$205^{-/-}$ mice were immunized with 10 μg of αDEC-205:LcrV mAb with maturation stimuli, and 2 wk later, splenocytes were restimulated with LcrV-reactive peptides (KILAYFLPEDA, aa 73–83). The percentage of IFN-γ^+ $CD4^+$ cells was assessed by intracellular cytokine staining. Shown is one representative experiment of two.

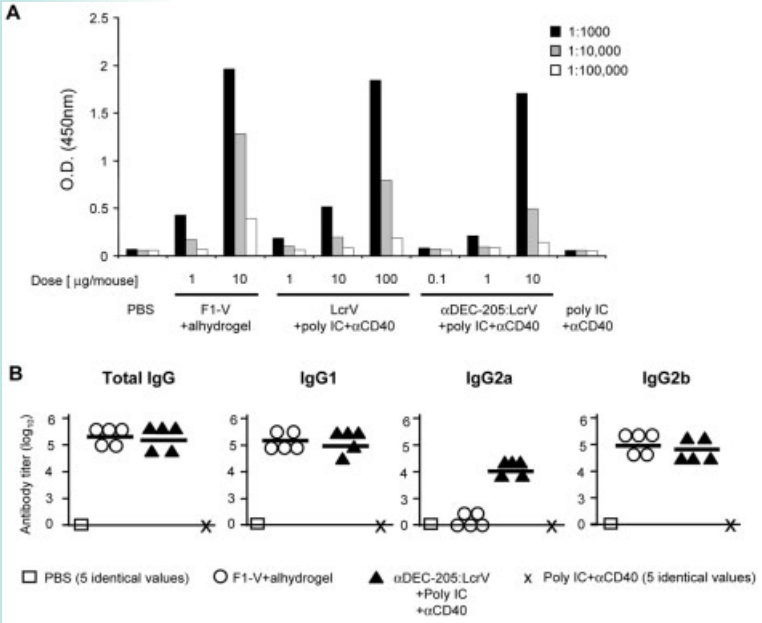

Figure 5. Antibody response following anti-DEC-205:LcrV (αDEC-205:LcrV) mAb immunization. (A) Anti-LcrV IgG antibody titers after 2 wk of i.p. injection with graded doses of F1-V adsorbed in alum, αDEC-205:LcrV mAb, and LcrV protein in the presence of adjuvants [50 μg of poly(IC) and 25 μg of anti-CD40], or adjuvants alone. At least, two similar experiments were repeated and one representative experiment is shown. (B) Anti-LcrV antibody titers for individual IgG isotypes after 2 wk of i.p. injection with PBS, F1-V (10 μg) adsorbed in alum, αDEC-205:LcrV (10 μg) mAb in the presence of adjuvants [50 μg of poly(IC) and 25 μg of anti-CD40], or adjuvants alone. Symbols represent individual mice, and the horizontal line represents the mean value of each group. Shown is one representative experiment of three.

Discussion

It is possible that a successful vaccine for plague needs to induce combined cellular and humoral immune responses against virulence determinants with minimal side effects. The need for cellular immunity was first indicated by the studies of Nakajima and Brubaker [15], who noted protective effects of exogenous IFN-γ and TNF-α. Elvin *et al.* [17] then reported that STAT4-deficient mice, which are only able to produce very low amounts of IFN-γ, were poorly protected by s.c. infection with *Y. pestis* even though they produce high levels of antibody similar to controls. A protective role of $CD4^+$ T cells in *Y. enterocolitica* infection was directly demonstrated by Smiley and colleagues [18], where three LcrV-specific CD4 epitopes were identified that are presented in the context of the murine $I\text{-}A^b$ MHC class II molecule. This group further showed that the transfer of *Y. pestis*-primed T cells to naive B cell-deficient mice (μMT) protected against attenuated *Y. pestis* (KIM D27) intranasal challenge [19]. Here we have addressed approaches to induce strong T cell immunity of the IFN-γ–producing Th1 type in otherwise naive mice.

Prior plague vaccines, such as killed whole-cell vaccine and attenuated vaccine, induce protection against bubonic plague, but the vaccines evoke severe side effects and do not provide protection against pneumonic plague [3]. A subunit vaccine composed of F1 and V proteins has provided the most promising efficiency based on the data obtained from small animal models [4]. Along with evidence showing protection through adoptive serotherapy, the general mechanism by which F1-V subunit vaccine provided protection is considered to be antibody-mediated, and especially, the IgG1 isotype antibody plays an important role [26, 27].

It has been known that *Y. pestis* targets immune cells during infection, and through its specialized delivery system or type III secretion system, it has a range of anti-host functions from anti-phagocytic activity to immunosuppression [28]. Therefore, to induce protective immunity, it may be valuable to activate immune cells such as DC and macrophages that are targeted by *Y. pestis*. Th1 cytokines might provide a way to bring about this activation and/or overcome the immunosuppression that is induced by *Y. pestis*. In addition, Th1-type immune responses can provide "help" for antibody-based immunity as well as the generation of memory responses [14, 29]. For these purposes, we have utilized DC and a DC-targeting strategy to induce immunity. We took an mAb against a specific DC receptor, anti-DEC-205/CD205 mAb, and genetically engineered the LcrV antigen of *Y. pestis* into the antibody heavy chain. Our data show the improved quality and quantity of T cell responses induced by DEC-205-targeted antigen.

Recently, LcrV protein epitopes recognized by CD4 T cells were identified in H-2^b and H-2^d mice in two independent studies [18, 30]. Since these studies depended on standard protein immunization for priming, high concentrations of protein or peptides or sometimes prime-boost strategies were required to induce detectable CD4 T cell responses. In addition, to detect immune responses, it was necessary to use either a proliferation assay with bone-marrow-derived macrophages and T cell hybridomas or ELISA for IL-2 released during *in vitro* restimulation with splenic antigen-presenting cells. Generating T cell hybridomas is time-consuming and also IL-2 is not the best reflection of Th1 cytokines.

In contrast, we assayed the immunity that was induced by *in situ* targeting of antigen to DC with intracellular cytokine staining of immune spleen cells challenged for 6 h with a peptide library of 15-mers spanning the full-length of LcrV protein. In this way we could document the presence of sizeable T cell responses with minimal tissue culture requirements. Our data showed that a single dose of smaller amounts of antigen conjugated to anti-DEC-205:LcrV mAb induced stronger IFN-γ-secreting $CD4^+$ T cell responses when compared to nontargeted soluble protein (Fig. 3A). In addition, relatively low doses (0.2 μM) of the mimetope peptides were used to activate the primed T cells in the assays (Fig. 3B).

We also could observe broad T cell immunity induced by a single dose of DC targeting, since we identified distinct CD4 T cell mimetopes in three different MHC haplotypes that were tested and at least two peptides in each haplotype (Fig. 2, Table 1, 2). We noted that the DC targeting strategy enhanced $CD4^+$ T cell responses relative to nontargeted soluble protein with standard adjuvants such as CFA or alhydrogel (Fig. 4A). Interestingly the peptides that were recognized following DEC-205-based immunization were identical to those reported by Parent *et al.* [18] and Shim *et al.* [30].

During primary immune responses, $CD4^+$ T cells control affinity maturation and isotype switching by antigen-specific B cells [31]. Cytokines secreted from the Th cells are important to determine isotype switching, such as IFN-γ (Th1) for IgG2a (BALB/c) or IgG2c (C57BL/6) production, or IL-4 (Th2) for IgG1, respectively [32]. As described in a previous study, DC targeting leads to a broad range of different antibody isotypes, or a combination of Th1 and Th2 responses [24]. In this study, we also observed that LcrV antigen targeted to DC induced multiple isotypes including IgG1, IgG2a/IgG2c, or IgG2b in serum (Fig. 5). However, F1-V recombinant protein vaccine could not induce Th1 isotype antibody as much as anti-DEC-205:LcrV mAb immunization, even though it generally leads to high titers of IgG1 and IgG2b isotypes.

Here we have used a combination of TLR3 ligand poly(IC) and agonistic anti-CD40 mAb as DC maturation stimuli to overcome T cell tolerance [25, 33–35]. Further study of active adjuvants is required. We observed that poly(IC) by itself did not lead to a primary immune response that was strong enough to be detected in our assays. We are now investigating the potential of poly(IC) alone as an optimal DC adjuvant in conjunction with a prime-boost strategy, and we are also interested in whether the $CD4^+$ T cell responses observed in the current study translate into a better quality and quantity of humoral immunity, especially memory. The contribution of the cellular immunity observed with our DC targeting strategy should also be investigated in protection studies, especially against an aerosol challenge of *Y. pestis*.

Materials and methods

Mice

C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice were purchased from Taconic, and DEC-$205^{-/-}$ mice (C57BL/6 background) are available from Jackson Laboratories. All mice were maintained under specific pathogen-free conditions and used at 5–7 wk of age according to the guidelines of our Institutional Animal Care and Use Committee.

Construction and production of fusion mAb and protein

The mammalian codon-optimized cDNA encoding LcrV (GenBank accession No. DQ917566) open reading frame of full length was generated by PCR, and was cloned in-frame with the C terminus of the heavy chain of mouse anti-DEC-205 mAb described in our previous study [25]. Anti-DEC-205:empty mAb is a mouse anti-DEC-205 mAb without any antigen insertion to the carboxyl terminus. The fusion anti-DEC-205:LcrV mAb and a control Ig:LcrV fusion mAb were produced by transient transfection into 293T cells using a calcium-phosphate method as described [25], and purified on Protein G column (Amersham Pharmacia Biotech). Soluble LcrV protein was generated by adding a signal peptide and FLAG epitope tag to the N terminus, expressed by stable transfection to CHO cells, and purified on anti-FLAG M1 affinity column (Sigma) according to manufacturer's protocol. A recombinant F1-V protein from U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Frederick, MD) has been described [36].

Immunization

Mice were injected intraperitoneally (i.p.) or s.c. in the hind footpads with fusion mAb, unconjugated anti-DEC-205 mAb, control Ig, or soluble LcrV protein in the presence or absence of adjuvants, which were 50 μg of poly(IC) with 25 μg of 1C10 agonistic anti-CD40 mAb per mouse. Soluble LcrV protein was also administered s.c. with CFA. F1-V recombinant protein was

administered with alhydrogel (2.0%; Superfos Biosector, Vedbaek, Denmark).

LcrV peptides

Fifteen-mer peptides spanning the entire LcrV sequence and overlapping by 11 aa were synthesized by the Proteomics Resource Center (The Rockefeller University). The library was divided into eight pools of 11 peptides except peptide pool 8, which was composed of ten peptides. The peptide pools spanned aa 1–46 (pool 1), aa 36–86 (pool 2), aa 76–128 (pool 3), aa 118–170 (pool 4), aa 160–210 (pool 5), aa 200–250 (pool 6), aa 240–291 (pool 7), and aa 281–326 (pool 8) of the LcrV protein.

Intracellular cytokine staining and surface staining

To detect LcrV-specific T cell immunity, spleen or lymph nodes cells were stimulated with pools of peptides (2 μM or indicated concentrations) or medium alone in the presence of a co-stimulatory anti-CD28 mAb (clone 37.51) for 6 h. Brefeldin A was added for the last 4 h to accumulate intracellular cytokines. Cells were then washed, incubated with anti-mouse CD3, CD4 or CD8 mAb for 20 min at 4°C after blocking Fcγ receptor with anti-CD16/CD23 antibody. Following fixation with Cytofix/Cytoperm Plus™ (BD PharMingen), cells were stained for intracellular IFN-γ or IL-2 for 15 min at room temperature. All mAb were purchased from BD PharMingen. Data were collected using FACSCalibur and analyzed by FlowJo (Tree Star).

ELISA for anti-LcrV antibodies

To detect LcrV-specific antibody, high-binding ELISA plates (BD Falcon) were coated with 10 μg/mL of LcrV protein overnight at 4°C. Plates were washed three times with PBS/0.1% Tween-20 and blocked with PBS/0.1% Tween-20/5% BSA for 1 h at 37°C. Serial dilutions of serum were added to the plates and incubated for another 1 h at 37°C. Various secondary goat anti-mouse Fc-specific antibodies conjugated with horseradish peroxidase (Southern Biotech) were then added and visualized with o-phenylenediamine (Sigma) tablet at room temperature for 15–30 min. The reported titers represent the highest dilution of sample showing an OD_{450} higher than 0.1, and the data are presented as the log antibody titer. Some of the data are shown as OD_{450} values.

Microscopy

Alexa488-conjugated anti-DEC-205:LcrV, or isotype control (mouse IgG1) antibodies were prepared using the Alexa Fluor® 488 protein labeling kit (Molecular Probes). A deconvolution microscope (Olympus America, Melville, NY), and one-, two- or three-color fluorescence labeling was used with antibodies and fluorochromes listed in the micrographs.

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Conflict of interest: R. M. Steinman is a consultant to Celldex, which is developing human DEC-205-based vaccines. Other authors have no commercial or financial conflict of interest.

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